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# Remarkably Distinctive Recognition of $\alpha$ -Alanine and $\alpha$ -Phenylalanine during the Water-Catalyzed Hydrolysis of an Activated Amide

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The rate of the water-catalyzed hydrolysis of three activated amides in aqueous solution is significantly retarded by small amounts of  $\alpha$ -phenylalanine as compared with the rate acceleration induced by other common  $\alpha$ -amino acids not containing a benzyl group in their side chain. These contrasting effects emphasize the large hydrophobicity of  $\alpha$ -phenylalanine and are of relevance for a better quantitative understanding of protein folding and molecular recognition processes involving proteins.

## Introduction

As part of our kinetic studies on molecular recognition processes in aqueous solutions,<sup>1</sup> we are quantitatively investigating noncovalent interactions between  $\alpha$ -amino acids (and simple derivatives) and activated amides, as mimics for the peptide bond. Quantitative data of this kind are of importance in obtaining insight into the interactions governing protein stability and the role of water therein.<sup>2</sup> There is strong evidence that hydrophobic interactions drive protein folding and determine to a large part protein stability.<sup>3,4</sup> However, the exact driving force for pairwise as well as bulk hydrophobic interactions is still a matter of considerable debate.<sup>5</sup>

In the present study, we have determined pseudo-first-order rate constants for the water-catalyzed hydrolysis of 1-benzoyl-3-phenyl-1,2,4-triazole (**1**) in the presence of several  $\alpha$ -amino acids at 25 °C and pH 4. The mechanism of this hydrolysis reaction (Scheme 1) has been elucidated in detail previously.<sup>6,7</sup> The hydrolysis is general base catalyzed, and at the experimental pH two water molecules are involved in the transition state with three protons in flight. The medium effects on this hydrolysis reaction arise from the large increase in substrate polarity during the activation process.

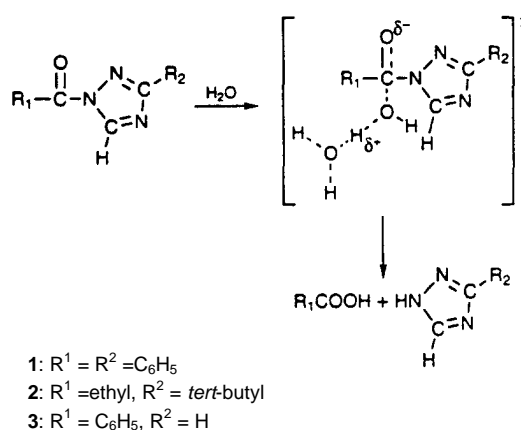
Using Savage and Wood's theory for the thermodynamics of solute-solute interactions in water,<sup>8</sup> the kinetics of the hydrolytic process can be quantitatively linked to a thermodynamic parameter ( $G(C)$ ) which reflects the difference in pairwise Gibbs energy of interaction of the cosolute with the initial state and transition state, respectively.<sup>9,10</sup>

$$\ln \left( \frac{k}{k^0} \right) = \frac{2}{RT} G(C) m_c - f n M_w m_c$$

In this equation,  $k$  and  $k^0$  are the pseudo-first-order rate constants in the mixed aqueous medium and pure water, respectively;  $m_c$  is the molality of the cosolute,  $f$  is the practical osmotic coefficient which equals one in highly dilute solutions,  $n$  is the number of water molecules involved in the transition state, and  $M_w$  is the molar mass of water. The second term of this equation reflects the influence of the cosolute on the reactivity of water.

In earlier studies this theory has been applied to a whole range of classes of cosolutes,<sup>1,11,12</sup> and solvent effects were interpreted

## SCHEME 1



primarily in terms of pairwise hydrophobic interactions of the cosolutes with the apolar initial state of the reaction.

## Experimental Procedure

$\alpha$ -Phenylalanine and  $\alpha$ -alanine were obtained from Janssen Chimica.  $\alpha$ -Phenylalaninamide and  $\alpha$ -alaninamide were purchased from Sigma. They were used without further purification. 1-Benzoyl-3-phenyl-1,2,4-triazole, 1-ethanoyl-3-*tert*-butyl-1,2,4-triazole, and 1-benzoyl-1,2,4-triazole were synthesized according to literature procedures.<sup>6</sup> Solutions were made up by weight using demineralized water which was distilled in an all-quartz unit and adjusted to pH 4 with an HCl solution.

Pseudo-first-order rate constants were determined by following the change in absorbance at appropriate wavelengths. Hereto about 5  $\mu\text{L}$  of a stock solution of the probe in acetonitrile was injected into 2.5 mL of reaction medium in a quartz cell and placed in a thermostated cell compartment (25.0 °C) of a Perkin-Elmer 15 or 12 spectrophotometer. Pseudo-first-order rate constants were calculated using a data station connected to the PE 15 and a fitting program in the case of the PE 12 spectrophotometer and were reproducible to within 2%.  $G(C)$  values were obtained from the slopes of the plots of  $\ln(k/k^0)$  versus  $m_c$  by linear regression. For the temperature dependent studies rate constants were measured over at least 20 °C and eight temperatures at a cosolute concentration of 0.15  $m$ . Plots of  $\ln(k/T)$  versus  $1/T$  were perfectly linear, and the enthalpies of activation were obtained from the slope whereas the entropies

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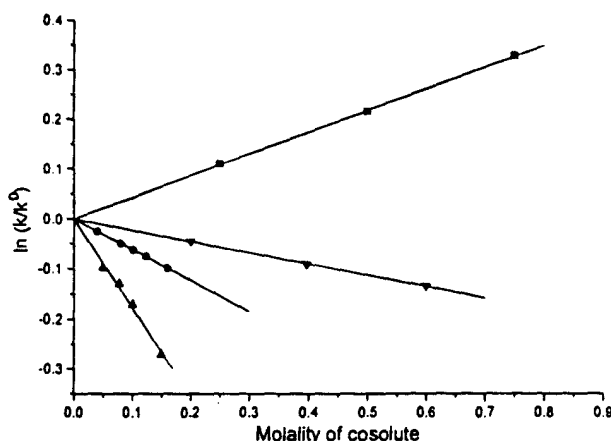
<sup>‡</sup> University of Leicester.

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**TABLE 1:  $G(C)$  Values for Several Cosolutes and the Kinetic Probes 1-3**

	$G(C)$ (J kg mol <sup>-2</sup> )		
	1 <sup>a</sup>	2	3
$\alpha$ -alanine	558 (16) <sup>b</sup>	693 (2)	478 (2)
$\alpha$ -phenylalanine	-709 (6) <sup>c</sup>	-1483 (185)	-502 (136)
$\alpha$ -alaninamide	-234 (6)		
$\alpha$ -phenylalaninamide	-1869 (25)		

<sup>a</sup> Compare, for example, the  $G(C)$  values for Gly (875 J kg mol<sup>-2</sup>) and  $\alpha$ -Val (467 J kg mol<sup>-2</sup>). <sup>b</sup> In water,  $\Delta^\ddagger G^\ominus = 89.65 \pm 0.02$  kJ mol<sup>-1</sup>,  $\Delta^\ddagger H^\ominus = 45.9 \pm 0.5$  kJ mol<sup>-1</sup> and  $\Delta^\ddagger S^\ominus = -147 \pm 2$  J mol<sup>-1</sup> K<sup>-1</sup>. In the presence of 0.15 M Ala,  $\Delta^\ddagger G^\ominus = 89.40 \pm 0.01$  kJ mol<sup>-1</sup>,  $\Delta^\ddagger H^\ominus = 47.40 \pm 0.3$  kJ mol<sup>-1</sup> and  $\Delta^\ddagger S^\ominus = -141 \pm 1$  J mol<sup>-1</sup> K<sup>-1</sup>. <sup>c</sup> In the presence of 0.15 M  $\alpha$ -Phe,  $\Delta^\ddagger G^\ominus = 89.78 \pm 0.01$  kJ mol<sup>-1</sup>,  $\Delta^\ddagger H^\ominus = 52.0 \pm 0.7$  kJ mol<sup>-1</sup> and  $\Delta^\ddagger S^\ominus = -127 \pm 1$  J mol<sup>-1</sup> K<sup>-1</sup>.



**Figure 1.** Plots of  $\ln(k/k^0)$  versus  $m_c$  for  $\alpha$ -alanine (■),  $\alpha$ -alaninamide (▼),  $\alpha$ -phenylalanine (●) and  $\alpha$ -phenylalaninamide (▲) on the pH-independent hydrolysis of **1**.

of activation were calculated from the Gibbs energy of activation at 25 °C and the enthalpy of activation.

An Omega titration microcalorimeter (Microcal, Northampton, MA) was used to obtain the changes in enthalpy accompanying the computer-controlled titration of 3-phenyl-1,2,4-triazole to  $\alpha$ -Phe, both in aqueous solution and at 30 °C and pH 4. The changes in enthalpy due to the dilution of 3-phenyl-1,2,4-triazole were subtracted from these data.

## Results and Discussion

We will focus our attention on the contrasting results obtained for aqueous solutions containing small amounts of the cosolutes  $\alpha$ -alanine and  $\alpha$ -phenylalanine, the sole difference being the replacement of a hydrogen for a phenyl ring in the  $\alpha$ -amino acid side chain. Both  $\alpha$ -amino acids are zwitterionic at pH 4.



$\alpha$ -Ala, R = Me;  $\alpha$ -Phe, R = CH<sub>2</sub>Ph

In accord with the results for a series of other  $\alpha$ -amino acids,  $\alpha$ -Ala increases the rate of amide hydrolysis with respect to the pure water reaction. By contrast,  $\alpha$ -Phe causes a large decrease in reaction rate. Quantitative results are collected in Table 1 and visualized in Figure 1. The ionized carboxylate of the  $\alpha$ -amino acid plays a major role in the rate accelerating effect, although no general base catalysis appears to be involved.<sup>13</sup> In the case of  $\alpha$ -Phe this effect is also operative ( $\alpha$ -phenylalaninamide causes an even larger decrease in reaction rate), but obviously there is a dominant opposing effect retarding

the hydrolytic process. Most likely the large deceleration caused by  $\alpha$ -Phe reflects pairwise hydrophobic interactions<sup>5</sup> between the apolar initial state of the reaction and the hydrophobic side chain of  $\alpha$ -Phe. The marked hydrophobicity of  $\alpha$ -Phe is well-documented in the literature.<sup>14</sup> We note that the presence of charges in the cosolute can complicate the interpretation of the medium effect,<sup>12,13</sup> and therefore we also investigated the medium effect of the uncharged benzyl alcohol, to ascertain that it is the benzyl moiety dominating the molecular recognition process. Indeed, benzyl alcohol retarded the hydrolysis even more than did  $\alpha$ -Phe, but a reliable  $G(C)$  value could not be obtained due to solubility constraints. Although it is known that *p-p* stacking interactions in water can take place<sup>15</sup> and that in proteins aromatic side chains often occur in clusters,<sup>16</sup> we contend that we can exclude the occurrence of these types of interactions in the case of  $\alpha$ -Phe on the basis of two observations. First, stacking interactions are not likely because rate retardations (Table 1) are also obtained for the hydrolysis of 1-ethanoyl-3-tert-butyl-1,2,4-triazole (**2**) a substrate slightly more hydrophobic than **1**.<sup>17</sup> Second, evidence for hydrophobic interactions playing a crucial role in the observed medium effects induced by  $\alpha$ -Phe is provided by measurements using the less hydrophobic kinetic probe 1-benzoyl-1,2,4-triazole (**3**). In this case the initial state stabilization is less for  $\alpha$ -Phe (a less negative  $G(C)$  value is obtained), which is in agreement with what one expects when hydrophobic interactions dominate the medium effect. In previous studies similar reduced medium effects for **2** in comparison with **1** were observed for nonaromatic cosolutes.<sup>17</sup> The  $G(C)$  value of  $\alpha$ -Ala changes only slightly (in the opposite way), suggesting a more complex interaction mechanism.

A final kinetic experiment was carried out in which  $\alpha$ -alaninamide was used as a cosolute in the hydrolysis of **1**. If hydrophobicity of the cosolute would be the sole determinant of the medium effect, then the difference in  $G(C)$  between  $\alpha$ -Ala and  $\alpha$ -alaninamide should be roughly the same as the difference in  $G(C)$  between  $\alpha$ -Phe and  $\alpha$ -phenylalaninamide (and the same holds for  $\Delta G(C)_{\alpha\text{-Ala}-\alpha\text{-Phe}}$  and  $\Delta G(C)_{\alpha\text{-alaninamide}-\alpha\text{-phenylalaninamide}}$ ). From Table 1 it is obvious that this is not the case, supporting the assumption that an analysis of the medium effects of  $\alpha$ -Phe,  $\alpha$ -Ala, and the other  $\alpha$ -amino acids solely in terms of hydrophobicity of the cosolute is a misinterpretation.

From the temperature dependence of the rate constants it became clear that the acceleration for  $\alpha$ -Ala is entropy driven, whereas the retardation observed for  $\alpha$ -Phe is enthalpic in origin (Table 1). A microcalorimetric titration experiment in which an aqueous solution of 3-phenyl-1,2,4-triazole (modeling the initial state) is titrated into an aqueous solution of  $\alpha$ -phenylalanine confirms the favorable enthalpic interaction between cosolute and initial state, thereby supporting the enthalpy driven retardation.

The present results demonstrate that noncovalent interactions with  $\alpha$ -amino acids in aqueous solution can be very side chain dependent,<sup>18</sup> an effect which has large consequences for protein folding and specificity of enzyme-substrate recognition.

We are currently investigating the medium effects of a whole series of  $\alpha$ -amino acids and derivatives on the hydrolysis of **1**.

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